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Issues in Biochemical Applications to Risk Assessment: When Can Lymphocytes Be Used as Surrogate Markers?

by George W. Lucier* and Claudia L. Thompson*

Introduction

During the course of this conference it has become clear that the application of *in vitro*, as well as *in vivo*, models for toxicity might be able to help us a great deal in our efforts to conduct more accurate species-to-species extrapolations of risk. However, the question remains: How do we monitor populations to determine if a particular biochemical pathway might have been perturbed or to determine if a particular population or individual might be sensitive or resistant to the actions of a given chemical?

In our attempts to address this question, the term "surrogate markers" has developed. These are markers which should be reflective of what's happening in the target cell or target organ. The most commonly used surrogate markers have been those that have measured concentrations of a chemical or its metabolites in body fluids or tissues, including urine, blood, and to a lesser extent, hair, milk, placenta, amniotic fluid, and fat. For the purpose of this discussion, it might be useful to classify them together as surrogate markers.

The basis for using markers in risk assessment and human monitoring reflects our growing knowledge of the mechanisms responsible for chemically induced disease. Figure 1 illustrates a general scheme for the sequence of biochemical events that occur between exposure and outcome. It is important to note that without knowledge of mechanism, it is not possible to differentiate between a marker of an exposure and a marker of an effect. Furthermore, the development of susceptibility markers also requires knowledge of mechanism. The appropriate use of susceptibility markers might offer an opportunity to identify individuals at risk and explain the huge interindividual variation in responsiveness to many chemicals. The use of the terms markers of exposure, markers of effect, and markers of susceptibility reflects the definitions used by a recent National Academy of Sciences panel (*1*).

*Laboratory of Biochemical Risk Analysis, National Institute of Environmental Health Sciences, P. O. Box 12233, Research Triangle Park, NC 27709.

The use of surrogate markers requires validation in humans. The validation is generally considered to consist of two processes. One is sensitivity and one is specificity. "Sensitivity" might be defined as the ability to adequately detect the nature of the chemical exposure of a given individual. When sensitivity is high, the occurrence of false negatives is low. Specificity reflects our ability to correctly classify individuals who are not exposed or who do not exhibit an adverse health effect. When specificity is high, false positives are low.

One approach that might be used in the development of surrogate markers requires the availability of adequate animal or cellular models for the toxic effects characteristic of the chemical being studied. In our opinion, the validation of surrogate markers is not possible without such *in vivo* or *in vitro* models. Validation requires characterization of the markers in that animal model throughout the time course of the disease process. It is then necessary to determine which of those markers can be determined noninvasively. And finally, this information must be applied to the human condition following either environmental, occupational, or medical exposures.

For the purpose of discussion, there are several different kinds of surrogate markers that have been used. These are listed in Table 1 and will be briefly summarized (*1–7*).

Chemical or Its Metabolites in Biological Media

The advantages of quantitating a chemical and/or its metabolites in body fluids is that it can provide a very specific marker for identifying exposure to well-characterized chemicals. Moreover, with recent advances in analytical chemistry, the current levels of detection offer increased sensitivity. However, there are some disadvantages to this procedure. Chemical detection will not reflect past exposure to rapidly metabolized chemicals. More importantly, levels of chemical in body fluids is not necessarily reflective of the interaction of the chemical with critical macromolecules in target cells.

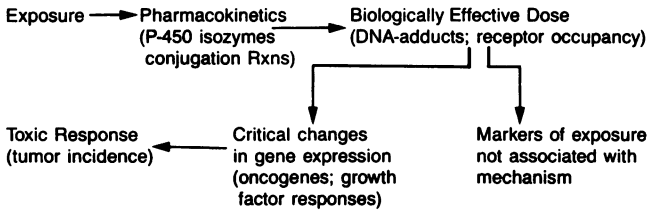

FIGURE 1. Mechanistic basis for the use of biomarkers in risk assessment and epidemiology.

Table 1. Surrogate markers.

Chemical or its metabolites in biological media
Mutagenic activity in body fluids
Cytogenetics
Gene mutations
Nucleic acid/protein adducts
DNA repair
Receptor interactions
Tumor markers

Mutagenic Activity in Fluids

Testing for mutagenic substances in urine obtained from chemically exposed persons has been a widely used monitoring technique. The use of this marker may be helpful in detecting exposures to complex mixtures when the identification of the toxicant is unknown. In its present state, these tests thus far have been qualitative rather than quantitative and are of limited value beyond possibly indicating that an exposure has occurred. This test is obviously not specific and usually not very sensitive.

Cytogenetics

Analyses of sister chromatid exchange (SCE) frequencies and chromosomal aberrations in peripheral human lymphocytes have been conducted in chemically exposed groups. The mechanism responsible for inducing SCEs is not understood; however, many classes of carcinogens and mutagens are known to produce increased SCE frequency. The significance of this increase in relation to disease outcome is unclear. Unlike SCE, which is an S-phase-specific phenomenon, chromosomal aberrations can occur at any point of the cell cycle. Moreover, there is increasing evidence which suggests that chromosomal aberrations might be linked to the carcinogenic process for some chemicals. Like tests for detecting mutagens in body fluids, cytogenetic analyses might be useful for detecting exposure to complex mixtures but are generally not specific and in many cases not very sensitive.

Gene Mutation

Unlike the previously described assays, gene mutation is the result of direct damage to DNA. Currently, somatic mutation of the HPGRT gene locus in human lymphocytes is being evaluated as a monitoring tool, as is the loss of an allele at the glycophorin A locus in erythrocytes. These assays have been used to detect exposure to cancer chemotherapy agents but have not successfully been applied to environmental exposures. They are in the early stages of development, but do offer the advantage for detecting current as well as past exposures. It remains to be seen what the relationship will be between elevated mutant frequencies at these sites and primary or secondary (in the case of chemotherapy patients) cancer.

An exciting approach for monitoring gene mutation that offers the opportunity to be both sensitive and specific is being developed. It involves enzymatic amplification of mutant gene sequences followed by analysis on gradient denaturing gels (*3*). This technique avoids the clonal expansion of mutant cells (as is necessary for HPGRT) by selective pressure and therefore might allow one to identify a broader spectrum of mutations.

Nucleic Acid and Protein Adducts

Several methods to detect DNA-chemical adducts in exposed persons are currently available. These include synchronous fluorescence spectrophotometry, detection by monoclonal or polyclonal antibodies, and ^{32}P postlabeling. The potential advantages of directly measuring DNA adducts is that it can be used as a measurement of the biologically effective dose and thereby take into account the interindividual variation in pharmacokinetic parameters. Monoclonal antibodies offer the advantage of being specific. The ^{32}P postlabeling method is extraordinarily sensitive and has the potential to detect 1 adduct in 10^{10} normal nucleotides. Unlike detection by monoclonal antibodies, which requires considerable knowledge of the DNA-chemical adduct and can only be used when appropriate antibodies are available, ^{32}P postlabeling picks up most bulky DNA adducts. However, there are some disadvantages with this assay. With current methodological procedures, it is only semiquantitative. In population monitoring studies, it should be noted that the level of DNA chemical adduct measured in peripheral lymphocytes may or may not be representative of levels found in target tissues.

There is increasing belief that hemoglobin adducts may be a good dosimeter of exposure for human populations. Steve Tannenbaum's work with the 4-amino biphenyl hemoglobin adducts (*8*) demonstrates that it is a quite sensitive marker to detect exposure to cigarette smoke, and perhaps other amines as well. Because of the longevity (120 days) of erythrocytes, some information can be obtained about past exposure. There is evidence that hemoglobin adducts may be good indicators of DNA damage; dose-response relationships are similar to those for DNA adducts for some chemicals. One of the disadvantages of this assay is that it requires expensive equipment, and, as with DNA adducts, adduct levels in hemoglobin may not accurately reflect actual exposure of target tissues.

DNA Repair

Methods to monitor DNA damage and/or repair in humans following chemical exposure include alkaline elution, unscheduled DNA synthesis, and nucleoid sedimentation. Though widely used in animal studies, these assays still require validation in humans. As markers of exposure, these assays generally lack both specificity and sensitivity. The potential strength of these assays may be there use as markers of susceptibility rather than markers of exposure. Repair deficiencies in individuals may be identified by exposing lymphocytes *in vitro* with different classes of chemical carcinogens. Nucleoid sedimentation assays may offer the advantage of detecting both repair deficiencies as well as damage.

Receptor Interactions

Inasmuch as many carcinogens appear to act through nongenotoxic mechanisms, markers need to be developed that reflect the complex nature of the interactions between receptor systems, signal transduction pathways (e.g., EGF, steroid receptors, protein kinases, TPA receptors), and regulation of cellular proliferation. If we could adequately measure receptor occupancy, for example, for estrogens or TCDD, perhaps we might have a good monitor of a biologically effective dose in humans following exposure either to environmental estrogens or to the toxic halogenated aromatics. The disadvantage of these assays is that tumor promotion involves several poorly understood stages, thus making it difficult to evaluate the relationship between altered receptor interactions and adverse effects.

Tumor Markers

Tumor markers have been used to a limited extent. Alpha-fetoprotein has probably been the most widely used marker and has detected early forms of liver cancer in some populations. The disadvantages of tumor markers is that they are detecting relatively late stages in the carcinogenic process. For the most part, they are neither specific nor sensitive.

Conclusion

In conclusion, unless we understand mechanism of action, the use of surrogate markers is limited to markers of exposure. Without animal models to verify dose-response relationships, they are also limited. However, the rapid advances in molecular biology may offer tractable approaches to develop tests that are both sensitive, specific, and more predictive of outcome. It, therefore, would seem prudent to collect samples from exposed populations for the purpose of conducting validated assays now and storing samples for assays under development that promise to have increased sensitivity and specificity.

Discussion

DR. ROY ALBERT, UNIVERSITY OF CINCINNATI MEDICAL CENTER: I'm not sure that you answered the question. When can lymphocytes be used as surrogate markers?

DR. LUCIER: Let's provide a basis for discussion. I think they can be used in a situation in which you have information about the mechanism and you have an adequate animal or *in vitro* model in which to verify your observations, especially dose-response relationships. I think everyone would agree that these kinds of observations can be very useful in detecting exposures, and they have been used to detect exposures. But what does it mean when you have a given concentration of a chemical in blood or an increase in sister chromatid exchanges? I'm not sure I understand what that means toxicologically. It may mean that these individuals have been exposed to a mutagenic agent. I don't know the general feeling of this audience. Nevertheless, biomarkers are going to be used increasingly as we develop molecular epidemiological approaches. The basis of these approaches is the availability of valid surrogate markers.

Unless we struggle with these markers (what they mean, what they're telling us), we're going to get ourselves into a situation where we have a pile of data that says, "Yes, this population may have been exposed, it may have been exposed more than the other one." But it's not going to tell us too much about outcome, unless we understand the toxicological basis of those observations. I think in some cases surrogate markers may be reflective of what's happening in the organ system of interest; in other cases they won't be. It seems to me that some sort of animal or *in vitro* model is needed to determine this.

UNKNOWN SPEAKER: One of the toughest problems is the issue of validation, for example, cytogenetic effects where you do have some reproducibility in methodology, at least these assays have been around for quite some time. How do you see going about validating the use of these as any index? Because you can't just look at those that have been exposed to hazardous substances. You've also got to accommodate the background frequency and exposures to nonhazardous substances that may have caused the same effect. I see validation as a real perplexing problem.

DR. LUCIER: The biggest problem with the validation of markers, I think, is tying them to toxic effects. The approach is to take a population, and say this population has been exposed to cigarette smoke. They have an overall increase in SCEs. But the increase in SCEs is very small, sometimes not even statistically significant. Yet you have a population that is going to go on and get a fairly high incidence of cancer. Whereas, in another case, another exposure might cause a greater increase in SCE frequency and the outcome might not be as severe. So I think the question of validation is a very difficult one because the quantitative relationship be-

tween the magnitude of the change and effect is far from clear.

I think we currently have some reliable markers of exposure and they may be good dosimeters in some cases. However, people who start talking about markers of effect, at this point, are whistling in the wind.

DR. RAYMOND TENNANT, NIEHS: I guess the point that you're making and that I tried to make before is in the interspecies extrapolation of data. These are the same types of parameters that one can measure in the short-term assays that I was discussing in my presentation. And I think we can put the best face on this in saying that although we don't understand how much mutagenic activity in the urine becomes significant to the person who is ever going to get a tumor, I think it is still significant that we measure this. And I think it is important to identify what is the mutagenic activity of certain compounds in human tissue or in human urine of exposed individuals. Because even though we don't understand the whole pathway right now, it's certainly worth the effort because the significance of these measurements might be more clear in the future.

DR. JULIAN PETO, ROYAL CANCER HOSPITAL, ENGLAND: Is there extractable and clonable DNA in frozen blood samples that you might be able to look at to estimate frequency of mutation or DNA adducts that might have been caused by chemical exposures?

DR. LUCIER: You certainly can look at DNA adducts. They're very stable. I don't know if frequency of mutations could be reliably estimated in these samples using current methodology.

DR. PETO: Could you clone the extractable DNA?

DR. LUCIER: I don't know if you could do that or not. Could anyone comment on that?

DR. PETO: You'd want to pull the same segment from two bits. You'd want to go to DNA twice in the same sample, pull the same sequence twice, and then compare them to see what sort of mutations were present. Maybe mutation is so difficult to estimate (vanishing) that it's not technically feasible. But it's the sort of thing that could be presumably done.

DR. LUCIER: The mutation frequency in exposed populations is probably very low, and some of the mutation assays are complicated by a varying baseline, such as the HPRT locus, so the low frequency coupled with the varying baseline and the fact that these samples might have been stored a long time suggests that it's probably not too practical at this time. However, some of the new techniques under development, such as amplification of mutant genes, may lead to approaches to detect low frequency mutations caused by chemicals.

DR. ALBERT: One of the problems in cancer epidemiology in industry is trying to reconstruct a past exposure, which can vary because of changes in processes or changes in jobs, and I wonder to what extent can the lymphocytes be used to reconstruct the magnitude of cumulative past exposure?

DR. LUCIER: Well, many lymphocytes live a long time, up to 3 years. So, it is possible to gather some cumulative information by looking at lymphocyte markers such as DNA adducts. However, quantitation of blood concentrations of a chemical or metabolite that's rapidly metabolized would not be useful in reconstructing past exposures. For looking at something like TCDD or the dibenzofurans or the PCBs, which are extraordinarily persistent, you can look several years later and get an idea of past cumulative exposure. So I think the point would be that you have to evaluate each marker, whether it be a DNA adduct or whether it be a hemoglobin adduct or DNA repair or receptor interaction coupled with knowledge of the persistence of the chemical and then make a determination as to whether or not it would be useful. So I don't think there is a unifying rule that would be useful. I think each chemical would have to be treated and each marker as a separate case.

UNKNOWN SPEAKER: What's the replicating capacity of lymphocytes when they're hit with a mitogen?

DR. LUCIER: It's very rapid, and that's how one analyzes for SCEs. When you hit lymphocytes with mitogens you induce a number of changes such as in the P-450 systems, which can metabolically activate any number of chemicals. So if you incubate benzo[a]pyrene with lymphocytes in the presence of a mitogen, you can get a lot of DNA adducts because the metabolic activation capacity is there in those lymphocytes, but that's not an *in vivo* monitoring situation. That's fine for *in vitro* perturbations of your system to evaluate markers of susceptibility, but this information does not necessarily provide a marker of effect.

DR. ALBERT: Have adducts in the brain been looked at? The brain contains neurons, which have essentially zero turnover, suggesting that they might be useful, although not as a noninvasive method. But in terms of autopsy or surgical material, the brain might provide a population measure of the buildup of adducts. What about adducts in the brain? Has that been looked at?

DR. LUCIER: In relation to animal models there's quite a bit of data on that. Adducts are found in the brain, usually found at a lower concentration, than many other tissues, although I don't know the structure-activity relationships. I don't know if adducts could be found in autopsy patients. The only way of looking at that would be through a postlabeling procedure or monoclonal antibody methods. You obviously wouldn't have treated that individual with a radioactive chemical to detect the DNA adduct. The method that Dr. Reddy talked about earlier (postlabeling) can possibly detect one adduct in a billion normal nucleotides. Perhaps by that method you might be able to detect some in those victims.

DR. ALBERT: You could have an occupational exposure that goes on for 30 years, the individual dies. You know what he was exposed to. Could you use monoclonal antibodies?

DR. LUCIER: Yes. You could use a monoclonal antibody to look at the adduct. But you have to remember there's some cross-reactivity with some of these, so you may get erroneous information unless you're careful. I would expect you could pick up adducts in the brain by the postlabeling procedure. They're picking them up in

virtually every tissue in control animals. So there's no reason why a so-called control human being wouldn't have them as well. But how you would relate that back to the exposure that had occurred I don't know, unless you had adequate controls to determine if those adduct spots were in fact different than the controls.

DR. ALBERT: I want to thank all the speakers and the participants for the discussion and close the session. Dave, do you want to give a benediction?

DR. HOEL, NIEHS: I want to thank everyone. I think this has been a successful conference. I certainly enjoyed many of the talks. I'm glad to see that a few people have lasted to the bitter end. And, I guess we will press hard to get manuscripts and get the publication out. I think the thing to do is to look for it in the *EHP*.

Thank you very much.

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